08/598874

# VACCINES AGAINST INTRACELLULAR PATHOGENS USING ANTIGENS ENCAPSULATED WITHIN BIODEGRADBLE-BIOCOMPATIBLE MICROSPHERES

## I. GOVERNMENT INTEREST

The invention described herein may be manufactured, licensed and used by or for governmental purposes without the payment of any royalties to us thereon.

## II. CROSS REFERENCE

This application is a continuation-in-part of U.S. Patent Application Serial No. 08/242,960 filed May 16, 1994, pending; which in turn is a continuation-in-part of ~~U.S.~~ Patent Application Serial No. 07/867,301 filed April 10, 1992, now U.S. Pat. 5,417,986 which in turn is a continuation-in-part of U.S. Patent Application Serial No. 07/805,721 filed November 24, 1991, now abandoned, which in turn is a continuation-in-part of U.S. Patent Application Serial No. 07/690,485 filed April 24, 1991, now abandoned, which in turn is a continuation-in-part of U.S. Patent Application Serial No. 07/521,945 filed May 11, 1990, now abandoned. Additionally, this application is a continuation-in-part of U.S. Patent Application Serial No. 08/446,149 filed May 22, 1995, pending, which in turn is a continuation of U.S. Patent Application Serial No. 06/590,308 filed March 16, 1984, now abandoned.

## III. FIELD OF THE INVENTION

This invention relates to parenteral and mucosal vaccines against diseases caused by intracellular pathogens using antigens encapsulated within biodegradable-biocompatible microspheres(matrix).

IV. Background of the Invention

Most infections by viruses and other intracellular pathogens are countered in the human host by a combination of humoral (antibody) and cellular (lymphocyte and phagocyte) immune effectors. Although the precise identity of immune effectors capable of protecting the host against some chronic intracellular pathogens (e.g. HIV-1) remains unknown, attempts to develop preventive and therapeutic vaccines still focus on the induction of appropriate humoral and cellular immune responses. Furthermore, since most human viral pathogens (including HIV-1) are transmitted across mucosal surfaces, it is important that vaccines induce such responses locally (at the mucosal surface) as well as systemically and that they be durable for long-term protection.

The issues of durability and mucosal immunogenicity have been previously addressed by encapsulating vaccine antigens in appropriately-sized biodegradable, biocompatible microspheres made of lactide/glycolide copolymer (the same materials used in resorbable sutures). It has been shown that such microspheres can be made to release their load in a controlled manner over a prolonged period of time and can facilitate the

interaction of their contents with the local immune system when administered mucosally.

In the case of HIV-1 infection, there is insufficient information at this time regarding the virus and its interactions with the human immune system to permit the rational design of a preventive vaccine. However, it has been noted that many candidate HIV vaccines tested to date fail to elicit antibodies capable of neutralizing wild-type HIV-1 or binding to native HIV-1 proteins, fail to induce a substantial population of effector cells capable of destroying HIV-1-infected cells, and fail to induce significant responses at mucosal surfaces. A possible approach to overcoming these problems (applicable to both HIV-1 and other chronic intracellular pathogens) is to identify a native protein, accessible to the immune system on the surface of both free virus and infected cells, and present it to the immune system (systemic and mucosal) encapsulated in microspheres to protect and augment its immunogenicity.

IV. Description of Drawings
Fig 1 indicates control Induction in Mice immunized with rgp 160; and
Fig 2 indicates "Native"/Denatured rgp 120 (IIIB) Binding Ratios

12/87

V. Description of the Invention

This invention relates to a novel pharmaceutical composition, a microcapsule/sphere formulation, which comprises an antigen encapsulated within a biodegradable polymeric matrix, such as poly(DL-lactide co glycolide) (PLG), wherein the relative ratio between the lactide and glycolide component of the PLG is within the range of 52:48 to 0:100, and its use, as a vaccine, in the effective induction of antiviral immune responses comprising both virus-

specific cytotoxic T lymphocytes and antibodies reactive against native viral antigens. In the practice of this invention, applicants found that when a complex (oligomeric) native envelope protein of HIV-1 was encapsulated in PLG microspheres, it retained its native antigenicity and function upon its release in vitro. Furthermore, when used as a vaccine in animals, this product elicited HIV-specific cytotoxic T lymphocytes and antibodies reactive with native (oligomeric) HIV-1 envelope protein.

The following examples illustrate the invention:

Example 1

Materials and Methods

*Immunogens.* Non-CD4-binding, baculo-expressed, recombinant $gp160_{IIIB}$ (rgp160) was obtained from MicroGeneSys (Meriden, CT). CD4-binding, oligomeric $gp160_{CDC451}$ (o-gp160) was obtained from Advanced BioScience Laboratories (Kensington, MD).

*Microencapsulation of immunogens*: PLG microspheres ranging from 1 to 20 um in diameter and containing a 0.5 to 1.0% antigen core load were prepared by a solvent extraction method. The solvent extraction method involves dissolving the viral antigen and sucrose (1:4 ratio w:w) in 1 ml of deionized water. This solution is flash frozen and lyophilized. The resulting antigen-loaded sucrose particles are resuspended in acetonitrile and mixed into PLG copolymer dissolved in acetonitrile. This antigen-polymer mixture is then emulsifyed into

heavy mineral oil, transferred into heptane and mixed for 30 min to extract the oil and acetonitrile from the nascent spheres. The spheres are harvested by centrifugation, washed three times in heptane and dried overnight under vacuum. Microsphere size was determined by both light and scanning electron microscopy. The antigen core load was determined by quantitative amino acid analysis of the microspheres following complete hydrolysis in 6 N hydrochloric acid.

*Analysis of immunogen spontaneously released from microspheres in vitro by binding to soluble CD4 and recognition by HIV-positive patient serum.* PLG microspheres loaded with native (oligomeric) gp160 were suspended in phosphate-buffered saline, pH 7.4 (PBS) , incubated at 37 C for 3 h, and then at 4 C overnight. The microspheres were then sedimented by centrifugation (2 min at 200 x g), the supernatants harvested, and the released gp160 assayed for binding to CD4 and recognition by HIV-positive patient serum by surface plasmon resonance (described below). A sample of the stock protein used for microencapsulation was assayed for comparison.

*Immunization of animals.* HIV-seronegative, 8-10 week old NZW rabbits were immunized intramuscularly with rgp160- or o-gp160-loaded PLG microspheres suspended in PBS or with alum-adjuvanted rgp160 in PBS. Groups receiving rgp160-loaded microspheres (n=2) were primed with 50 ug of immunogen on day 0 and boosted with 25 ug on day 42. Groups receiving o-gp160-loaded microspheres (n=3) were primed with 70 ug of immunogen on day 0 and boosted with 35 ug on day 56. Groups receiving alum-adjuvanted rgp160 (n=2) got 85 ug of immunogen on days 0, 7, and 28.

BALB/c mice were immunized subcutaneously with rgp160-loaded PLG microspheres suspended in PBS or with alum-adjuvanted rgp160 in PBS. The mice in all groups (n=4) received 10 ug of immunogen on days 0 and 21.

*Determination of the ratio of antibody binding to "native"/denatured rgp120IIIB measured by surface plasmon resonance (SPR).* Real-time binding interactions between ligand (gp120 covalently linked to a biosensor matrix) and ligate (Abs in solution) were measured using surface plasmon resonance (BIAcore, Pharmacia Biosensor, Piscataway, NJ). "Native" rgp120(IIIB) (Genentech, South San Francisco, CA) or reduced, carboxymethylated (denatured) rgp120(IIIB) (Genentech) was covalently linked to the biosensor dextran matrix. Sera and mAbs were diluted in HBS running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.05% (BIAcore) surfactant P20, pH 7.4) and injected through the dextran matrices at a flow rate of 5 ul/min. The binding value of each serum or mAb was measured in resonance units (RU), and the "native"/denatured gp120 ratios were determined by dividing the corresponding RU values and correcting for small differences in matrix concentration. Controls included an HIV-positive patient serum and mAb 1c1.

*Assessment of HIV-specific cell-mediated immunity in immunized mice by secondary CTL assay.* The spleens of BALB/c mice immunized on days 0 and 21 were harvested and single cell suspensions prepared aseptically in complete RPMI medium on day 35. The cells were then pooled within experimental groups (n=4), underlay with ficoll, centrifuged 30 min at 450 x g (RT), washed, and resuspended in complete RPMI medium. Following a 1 h stimulation with peptide p18 (1 uM) at 37ºC, the cell suspensions were diluted with complete RPMI supplemented with 2ME (1:1000) and transferred to flasks for a 6 day

incubation at 37°C. After 2 days, recombinant IL-2 (10 u/ml) was added to all flasks. On day 6, P815 target cells were pulsed with peptide p18 (1 uM) or with nothing (control) in PBS supplemented with 0.1% BSA. 3 x 10^6 target cells were labelled with 300 uCi of $^{51}Cr$, washed, and plated out with the effector cells at effector:target (E:T) ratios of 45:1, 15:1, 5:1, and 1.7:1. After a 6 h incubation at 37°C, the supernatants were harvested and counted, and % specific lysis was calculated.

Results

Comparison of the native (oligomeric) gp160 prior to microencapsulation and following spontaneous release from PLG microspheres showed the two to be essentially indistinguishable in terms of their binding to CD4 and recognition by HIV-positive patient serum (Table 1). This retention of conformation-dependent binding shows that structure of the antigen is not appreciably altered by the microencapsulation process.

Fig. 1 shows the data from a cytotoxic T lymphocyte (CTL) assay performed on the speen cells of mice which had had been previously immunized with either HIV-1 envelope protein encapsulated in PLG microspheres (dark squares) or the same protein administered in a conventional way with alum adjuvant (dark diamonds). These data indicate that microencapsulation of HIV-1 envelope protein in PLG microspheres results in a vaccine that induces significantly greater anti-HIV CTL activity than does alum-adjuvanted vaccine. The open symbol groups represent controls run to assure that the activity being measured is virus-specific.

Fig. 2 shows the results of an assay designed to measure the relative binding of antibodies to native vs denatured viral protein. These data show that rabbits immunized with a non-native HIV-1 protein encapsulated in PLG (#5 and 6) develop antibodies which show greater binding to denatured (vs native) protein (indicated by a ratio < 1). On the other hand, rabbits immunized with a native HIV-1 protein encapsulated in PLG microspheres (#10-12) develop antibodies which show greater binding to native viral protein (indicated by ratio > 1). This retention of each proteins antigenicity constitutes an additional piece of evidence that the structure of antigens loaded in PLG microspheres are preserved.

Example 2

Materials and Methods

This experiment was similar to that described in Example 1 except for the method of microencapsulation employed.

*Microencapsulation of immunogens*: PLG microspheres ranging from 1 to 15 um in diameter and containing a 0.5 to 1.0% antigen core load were prepared by a solvent evaporation method. The solvent evaporation method involves emulsifying the viral antigen dissolved in deionized water into poly(DL-lactide-co-glycolide) polymer dissolved in methylene chloride. This emulsion is mixed into 0.9% polyvinyl alcohol and stirred. After 10 min of stirring, 0.35 l of water is added and gentle mixing is continued for 1.5 h. The resulting

spheres are harvested by centrifugation, washed three times in distilled water, and dried overnight under vacuum. Microsphere size was determined by both light and scanning electron microscopy. The antigen core load was determined by quantitative amino acid analysis of the microspheres following complete hydrolysis in 6 N hydrochloric acid.

Results

Analysis of spontaneously released antigen showed it to retain its CD4 binding capacity. Its native antigenicity (recognition by the serum of an HIV-positive patient) was only slightly less than that of the antigen prior to encapsulation and following spontaneous release from microspheres produced by a solvent extraction method (Table 1).

The results of immunizing animals with either non-native (denatured) or native oligomeric gp160in PLG microspheres produced by a solvent evaporation method were essentially indistinguishable from those obtained using microspheres produced by a solvent extraction method (example 1). Microencapsulated antigen induced significantly greater CTL activity than antigen administered in a conventional alum-adjuvanted formulation. Furthermore, preservation of the structure of PLG-microencapsulated antigens is supported by the findings of preferential binding of antibodies elicited by microspheres loaded with denatured antigen to denatured gp120 (Fig. 2, #3 and 4) and the preferred binding of antibodies elicited by microspheres loaded with native (oligomeric) antigen to native gp120 (Fig. 2, #7-8).



10

Table 1

BIA (released o-gp160)

| Capture o-gp160-451 (stock vs microsphere-released) on tvc 391 fc3/fc4 sCD4 (4 mg/m | | | | |
|---|---|---|---|---|
| 1 ul/min flow rate for o-gp160 inj.; 5 ul/min for all others | | | | |
| | | | | |
| **Ligate** | **RU** | **HIV+/sCD4 (RU ratio)** | | |
| | | | | |
| gp120-MN 1:10 | 3286 | | | |
| HIV+ 1:100 | 54 | | | |
| NHS 1:100 | 3 | | | |
| HIV+ pool 1:100 | 47 | | | |
| | | | | |
| o-gp160 (tvc281) | 1772 | | | |
| HIV+ | 3259 | 1.84 | | |
| tvc281 | 1848 | | | |
| NHS | -38 | | | |
| tvc281 | 1762 | | | |
| HIV+ pool | 2597 | 1.47 | | |
| | | | | |
| tvc281-PLG-EV | 3342 | | | |
| HIV+ | 4594 | 1.37 | | |
| tvc281 | 3222 | | | |
| NHS | 7 | | | |
| tvc281 | 3210 | | | |
| HIV+ pool | 3336 | 1.04 | | |
| | | | | |
| tvc281-PLG-EX | 1855 | | | |
| HIV+ | 3780 | 2.04 | | |
| tvc281 | 1839 | | | |
| NHS | 2 | | | |
| tvc281 | 1850 | | | |
| HIV+ pool | 2745 | 1.48 | | |
| | | | | |
| | | | | |
| gp120-MN 1:10 | 2914 | | | |
| HIV+ 1:100 | 14 | | | |
| NHS 1:100 | -2 | | | |
| HIV+ pool 1:100 | 14 | | | |
| | | | | |
| tvc281 | 1099 | | | |
| HIV+ | 1083 | 0.99 | | |
| tvc281 | 1022 | | | |
| HIV+ pool | 1395 | 1.36 | | |
| | | | | |
| tvc281-PLG-EV | 1595 | | | |
| HIV+ | 1322 | 0.83 | | |
| tvc281 | 1535 | | | |
| HIV+ pool | 1781 | 1.16 | | |